TITLE OF PROJECT: RNA-INTERFERENCE AND CONTROL OF THE GLASSY-WINGED SHARTSHOOTER (*HOMALODISCA VITRIPENNIS*) AND OTHER LEAFHOPPER VECTORS OF *XYLELLA FASTIDIOSA*

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ABSTRACT: RNAi is a natural biological activity for controlling gene expression and for anti-viral defense in a majority of eukaryotic organisms, including insects. The application of RNA interference (RNAi) directed toward different types of insect plant pests is becoming more feasible and promising. RNAi is already used in commercial agriculture for plant virus control, and the many new publications demonstrating experimental successes with various plant-feeding insects suggest that RNAi could have a role in helping to manage Pierce's Disease of grapevines. In our efforts, we evaluated several approaches in attempts to induce RNAi effects in *H.*

vitripennis, the glassy-winged sharpshooter, an important vector of *Xylella fastidiosa*, the causal agent of Pierce's Disease of grapevines. In order to identify promising RNAi targets we performed transcriptome and small RNA next generation sequencing of *H. vitripennis*. We identified RNAi-based responses to *H. vitripennis*-infecting viruses and assessed in vitro feeding and transgenic plant assays as a means to initiate RNAi effects against *H. vitripennis*. We were able to demonstrate RNAi-induced decreased mRNA levels for specific RNA targets but we did not obtain consistent phenotypic effects on *H. vitripennis*.

LAY PERSON'S SUMMARY OF RESULTS: This work presents fundamental efforts towards understanding the feasibility of applying RNA interference (RNAi), to help combat Pierce's Disease of grapevines. Pierce's Disease is a significant threat to grape production in California and other parts of the U.S., and the causal agent, *Xylella fastidiosa*, a xylem-limited bacterium, also causes several other extremely important plant diseases worldwide. Our effort here does not directly target *Xylella fastidiosa*, but instead targets one of its most significant insect vectors, the Glassy-winged sharpshooter, *Homalodisca vitripennis*, and other sharpshooter vectors of *X. fastidiosa*.

We focused our efforts towards understanding and optimizing the means to induce RNAi effects in *H. vitripennis*. In this regard we evaluated specific interfering RNAs via in vitro assays and transgenic plant-based approaches. We also generated large scale genomic data along with transcriptome and small RNA datasets, to help us design rational and effective genetic/genomic efforts against *H. vitripennis*. We achieved target mRNA reductions in some assays but did not consistently induce desired phenotypic effects in recipient *H. vitripennis*.

INTRODUCTION: Our primary objectives were to evaluate and demonstrate RNA interference (RNAi) activity against *Homalodisca vitripennis* or the Glassy-winged sharpshooter (GWSS). GWSS is an important vector of *X. fastidiosa* and unlike other native sharpshooters, GWSS readily feeds on grapes and has the potential to move through vineyards moving *X. fastidiosa* as it feeds. New, environmentally sound approaches to target GWSS and other sharpshooter vectors of *X. fastidiosa* are needed in order to help manage Pierce's disease. RNAi strategies have the potential to help in long term, environmentally sound strategies to manage insects.

RNAi is a natural gene regulation and anti-viral defense mechanism found in insects and other organisms. RNAi was discovered in the early 1990's when studies with plants demonstrated that transgene-encoded RNAs did not accumulate in plants as expected, but were degraded in the cell cytoplasm in a sequence-specific manner (Jorgensen et al., 1996; Lindbo and Dougherty, 1992; Lindbo et al., 1993; Napoli et al., 1990). Most important from a practical sense is that these also correlated with desirable phenotypic effects in the plants. Since these initial findings, RNAi has become one of the most intensely studied areas in all of biology and the 2006 Nobel Prize in Physiology or Medicine was awarded for seminal mechanistic studies on RNAi in the nematode, *C. elegans* (Fire et al., 1998).

Double-stranded RNAs (dsRNAs), or single-stranded RNAs (ssRNAs) with significant intramolecular base paired regions, are recognized as powerful inducers of RNAi. These RNAs are processed by dsRNA-specific endonucleases (Dicers and/or Drosha, depending on the organism and cellular location) to yield small dsRNAs ranging from 20 – 30 bp. The resulting small dsRNAs are unwound and one strand (the guide strand) is incorporated into the Argonaute 1 (AGO1)-associated RNA-induced silencing complex (RISC). When the guide RNA searches and finds a complementary ssRNA, RNAi activity results, either mRNA degradation or interference with mRNA translation, depending on the type of guide RNA and the amount of base-paring with the RNA target. Although RNAi processes vary in different organisms, the overall mechanisms among various eukaryotes are generally conserved (Siomi and Siomi, 2009). We use the term "siRNAs" to refer in general to the 21 – 25 nt small RNAs generated by RNAi activity. The major value of RNAi approaches for agriculture is that they can be used to very selectively reduce specific gene expression and induce desirable phenotypes in plants, particularly to prevent pathogen or pest attack.

Transgenic RNAi-specific approaches have already been demonstrated to be very effective for many different plant viruses, and commercial RNAi-based antiviral resistance is used in U. S. papayas, squash and recently in plums (Fuchs and Gonsalves, 2007; Gonsalves, 2006; Scorza, 2013; Tricoli, 1995). Hundreds of thousands of these plants have been planted in the U. S. and the specific, RNAi-based anti-viral resistance has proven to be robust and to provide environmentally sound virus disease control with no identified negative effects. In recent years RNAi has been evaluated as a control strategy for insects, even leading to the suggestion of "insect-proof plants" (Gordon and Waterhouse, 2007). In fact, a recent issue of *Science* (16 August 2013) featured on the cover the suggestion of "smarter pest control" and a special section within that issue (pages 728 – 765) was dedicated to our "pesticide planet" (Kupferschmidt, 2013), and how new opportunities based on our understanding of RNAi could help feed the world's growing population and how this could be done in a way that improves sustainability in agriculture by decreasing our dependence on pesticides for pest control. We took fundamental approaches to assess the potential for inducing RNAi effects in GWSS and evaluated different strategies to induce RNAi activity. We successfully demonstrated induction of RNAi effects by several approaches, but did not consistently demonstrate the ability to induce negative phenotypic effects in *H. vitripennis*. However, our results provide new information that is important for assessing RNAi strategies against insect vectors of plant pathogens.

OBJECTIVES:

I. To generate and evaluate transgenic potato plants for their ability to generate small RNAs capable of inducing RNAi effects in *Homalodisca vitripennis*.

II. To identify GWSS interfering RNAs for practical application.
a) To utilize transgenic potato plants as efficient alternatives for identifying, delivering, and evaluating efficacious interfering RNAs.
b) To enhance production of interfering RNAs *in planta*.

III. Generate and use microRNAs from different developmental stages of GWSS insects.

IV. Assess the potential of using plant viruses for delivery of small RNA effectors.

RESULTS AND DISCUSSION:

Objective I. RNAi in *H. vitripennis* cells and insects. Initially, we used 14 GWSS Genbank cDNA sequences corresponding to known proteins in order to synthesize RNAi inducer molecules, dsRNAs. We then tested whether RNAi was inducible in GWSS cells and insects, and we showed that RNAi activity is inducible in GWSS (Rosa et al., 2010). Quantitative RT-PCR, semi quantitative RT-PCR, and Northern blot of small and large RNA fractions showed that RNAi was achieved in cells and insects injected with dsRNA where target mRNAs were partially degraded and specific siRNAs (short-interfering RNAs), hallmarks of RNAi, were detected (Rosa et al., 2010).

Table 1: GWSS insect sequences used for cloning and generation of potato transgenic lines.

GWSS Targets	Potato Pedigree	Potato Variety	Selection Method	Small RNAs
Chitin Deacetylase	102203	Kennebec	Basta	yes
Chitin Deacetylase	102203	Kennebec	Basta	yes
Actin	112064	Desiree	Basta	yes
Cuticle	102203	Desiree	Basta	yes

Generation of transgenic plant lines. In order to generate dsRNAs that can target GWSS, target sequences were cloned into a gateway-compatible binary vector pCB2004B. The target sequences were cloned in head to tail direction in the gateway vector with a non-homologous sequence between them. Upon transcription in transgenic plants, these constructs will yield double-stranded, hairpin RNAs of the desired sequence. The expression vectors carrying the insect target sequences of interest were first cloned into *E.coli* and *Agrobacterium tumefaciens* and they have been sequence verified. *A. tumefaciens* cultures carrying the sequences of interest were used to transform potato plants (Kennebec and Desiree varieties) against GWSS target genes Actin, Cuticle and Chitin Deacitilase (Table 1). Transformation / regeneration were performed via recharge at the UC Davis Ralph M. Parsons plant transformation facility (http://ucdptf.ucdavis.edu/) and approximately ten independent transgenic lines were obtained for each of the constructs. We performed screening of these transgenic potato plants for insert composition and generation of small RNAs (Fig. 1). We vegetatively propagated the T_0 plants confirmed to yield the desired RNAs for use in RNAi experiments with GWSS. In addition to the transgenic plants expressing GWSS target genes under control of the 35S promoter we generated some potato plants with transgene expression under a specific xylem promoter EgCAD2, which was cloned from *Eucalyptus gunii*.

Objective II. We compared transgenic potato plants engineered to express interfering RNAs to target GWSS in RNAi feeding assays. We used plants with transgenes driven by two different promoters for these experiments, the 35S constitutive promoter and the EgCAD promoter from *Eucalyptus gunii*. These assays showed that we were able to induce RNAi effects in GWSS as determined by RT-qPCR analysis of target mRNAs (Fig. 2), but we failed to generate a detectable phenotype on the GWSS, all looked normal and we observed no mortality different from GWSS fed on non-transgenic control plants. We now believe that this may be due at least in part because of how we performed our assays. We believe that plant-based RNAi-induced phenotypic effects are more dramatic on nymphs that develop on the plants expressing RNAi inducers.

We used potato cuttings with caged 4th and 5th instar GWSS nymphs. The cuttings were placed in dilute nutrient solution and GWSS remained on cuttings for ~7 days (Fig. 3). The GWSS nymphs were allowed to feed on the cuttings for 5 days at which point the insects were harvested and RNA was extracted to test for target mRNA knockdown using RT-qPCR. Unfortunately, these feeding trials did not induce a detectable phenotype or result in consistent, detectable reduced target gene expression when compared to the wild type and GFP negative control

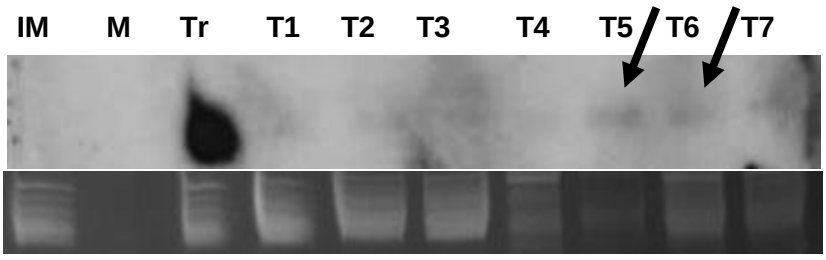

Figure 1. Small RNA northern hybridization analysis of GWSS-Actin transgenic potato plants. Arrows indicate positions of GWSS anti-actin siRNAs. Lower intensity siRNA signals are present in many of the other lines.

plants. Our ongoing efforts with phloem-feeding hemipterans have shown similar results, but we have been able to see negative phenotypes only when we allow target insects to develop on test plants, they must go through nymphal instar stages and molt. For GWSS this a little problematic as they like to move among plants and feed on different species, in fact in order to have sufficient reproduction we rear them in cages containing basil, cotton and cowpea plants. We also performed experiments using 2nd and 3rd instar nymphs and kept insect until they became adults, but no obvious effects were observable.

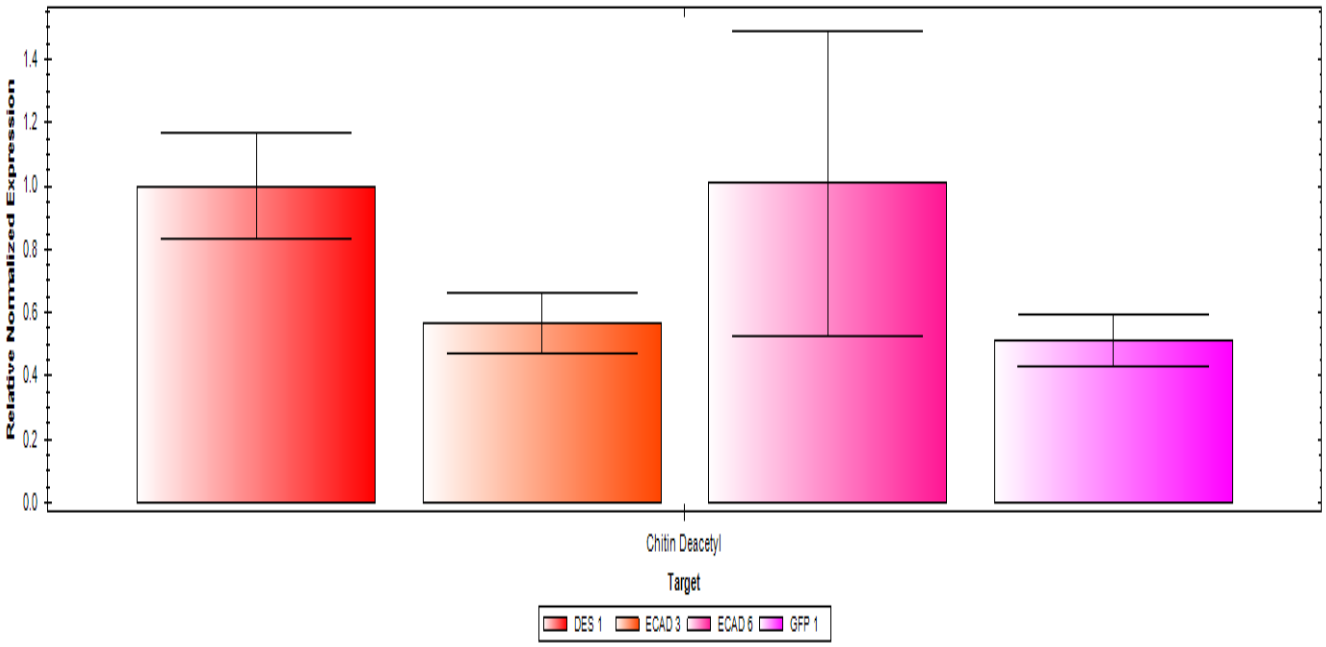

Figure 2. Relative normalized expression of the GWSS chitin deacetylase gene after GWSS feeding on wild type and transgenic plants expressing dsRNA showing no difference in target gene expression between wild-type and transgenic plant lines. DES 1 is the wild type potato control. ECAD 3 and ECAD 6 are separate transgenic lines expressing dsRNA for GWSS chitin deacetylase under control of the EgCAD promoter. GFP 1 is a control transgenic line expressing dsRNA for GFP. The GWSS ubiquitin gene was used as an internal control for the RT-qPCR. Error bars represent the standard error of the data.

Figure 3. GWSS feeding on basil stem which is submerged in a solution of double-stranded RNA.

Objective III. We evaluated three approaches for expressing artificial microRNAs (amiRNAs) in plants. Our intent was two-fold: one is to use specific amiRNAs to target GWSS mRNAs and reduce the possibilities for potential RNAi off-target effects which are more possible with longer, dsRNA RNAi inducers (Nunes, 2013); and

second, we have identified several GWSS-novel miRNAs by Illumina-based sequencing and bioinformatics analysis (see Fig. 4). We have so far only identified miRNAs in adult GWSS, but our goals are to identify potential miRNAs that may be GWSS instar-stage specific and evaluate their potential for use in RNAi towards GWSS.

We used agroinfiltration of *N. benthamiana* plants, followed by small RNA hybridization and Illumina sequencing to assess production of amiRNAs. These experiments showed that we can produce specific amiRNAs in plants by two methods: one by using a binary plasmid vector to produce the specific amiRNA; and second by using a modified begomovirus A component to replicate and express higher levels of amiRNAs in plants. The latter suggests that it is worth investigating using *Grapevine red blotch associated virus* (GRBaV) (Krenz et al., 2014) as a means for generating specific amiRNAs in grapevines.

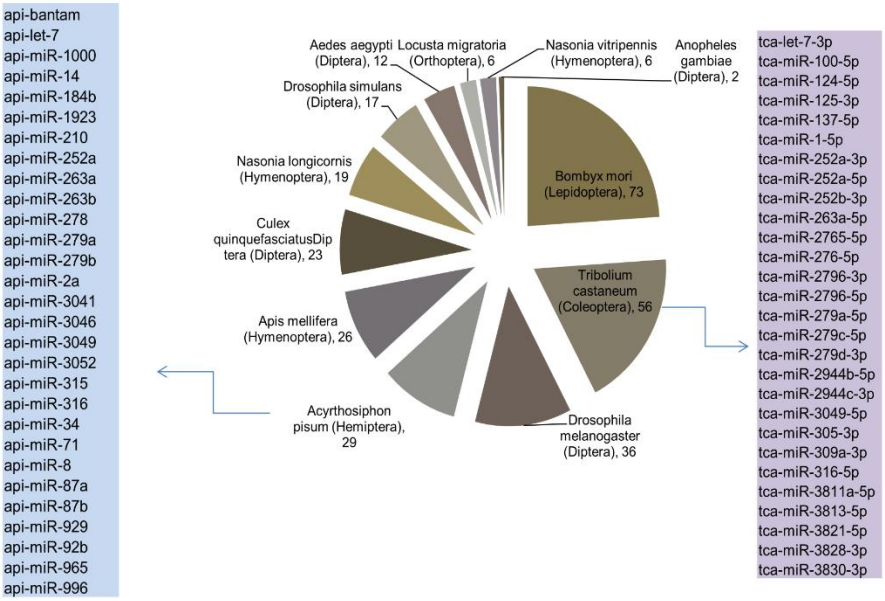

Figure 4. The microRNA profile analysis of GWSS adult insects revealed the presence of microRNAs that are conserved between different insects. GWSS adults also share some microRNA conservation with plants.

Objective IV. Our efforts here were based on our previous successes using plant-infecting viruses to express interfering RNAs in plants. There we used recombinant plant viruses expressing insect RNAi inducers and were able to achieve negative phenotypes in specific phloem-feeding target insects. Here we attempted to engineer *Grapevine leafroll-associated virus-7* (GLRaV-7), a phloem-restricted virus from the complex family *Closteroviridae*, and *Grapevine Red blotch-associated virus* (GRBaV) for our studies. This is based on successes by others using viruses from the same family such as *Citrus tristeza virus* in citrus (Dawson and Folimonova, 2013; Folimonov et al., 2007), and GLRaV-2 in grapevines (Dolja and Koonin, 2013). In both cases the plant virus-based vectors were capable of regulating the expression of endogenous genes via virus-induced gene silencing in their respective host plant, and were capable of expressing foreign genes/sequences for long periods of time showing a significant stability and durability. Furthermore, our own results using a DNA virus-based system (unpublished) showed that we were able to produce specific artificial micro RNAs in plants.

We have a GLRaV-7 isolate from California in culture (Genbank accession number: JN383343; Al Rawhnih et al., 2012), and now have generated complete, full-length cloned cDNAs for this virus. The entire cDNA of GLRaV-7 is now cloned into a binary vector, pCAMBIA$_{1380}$. Experiments are still ongoing to assess infectivity by

using standard agroinfiltration, but we also will attempt using vacuum infiltration of grapevine plants as it was successfully reported for GLRaV-2 in grapevines (Kurth et al, 2012).

Based on our success with expressing amiRNAs in plants we also have been attempting to generate infectious cloned cDNA versions of GRBaV. We are making progress but this work also is ongoing.

CONCLUSIONS: RNAi is a natural biological activity for controlling gene expression and for anti-viral defense in a majority of eukaryotic organisms, including insects. The application of RNAi directed toward different types of insect plant pests is becoming more feasible and promising. In our efforts, we were able to induce RNAi effects in *H. vitripennis* and evaluated initial transgenic plants as a means to initiate RNAi effects in the glassy winged sharpshooter and other leafhopper vectors of *Xylella fastidiosa*. Our lack of producing a desired negative phenotype in target *H. vitripennis* was due at least in part to the experimental system and biology available. As we have learned more about *H. vitripennis* and RNAi application potential, it seems unlikely that the plant-based approaches used by us have good potential for helping to manage *H. vitripennis* and other sharpshooter vectors of *X. fastidiosa*. This is important and thus our data are useful for long term decisions. However, because RNAi effects can be induced in *H. vitripennis*, the use of insect-infecting viruses modified to induce specific RNAi effects in sharpshooters is a potential strategy that could be considered for future experimentation.

REFERENCES CITED:

Al Rwahnih, M., Dolja, V.V., Daubert, S., Koonin, E.V., Rowhani, A., 2012. Genomic and biological analysis of *Grapevine leafroll-associated virus 7* reveals a possible new genus within the family Closteroviridae. Virus Res. 163, 302-309.

Dawson, W.O., Folimonova, S.Y., 2013. Virus-based transient expression vectors for woody crops: a new frontier for vector design and use. Annual review of phytopathology 51, 321-337.

Dolja, V.V., Koonin, E.V., 2013. The closterovirus-derived gene expression and RNA interference vectors as tools for research and plant biotechnology. Frontiers in microbiology 4, 83.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806-811.

Folimonov, A.S., Folimonova, S.Y., Bar-Joseph, M., Dawson, W.O., 2007. A stable RNA virus-based vector for citrus trees. Virology 368, 205-216.

Fuchs, M., Gonsalves, D., 2007. Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. Annual review of phytopathology 45, 173-202.

Gonsalves, D., 2006. Transgenic papaya: development, release, impact and challenges. Adv Virus Res 67, 317-354.

Gordon, K.H., Waterhouse, P.M., 2007. RNAi for insect-proof plants. Nature biotechnology 25, 1231-1232.

Jorgensen, R.A., Cluster, P.D., English, J., Que, Q., Napoli, C.A., 1996. Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant molecular biology 31, 957-973.

Krenz, B., Thompson, J.R., McLane, H.L., Fuchs, M., Perry, K.L., 2014. Grapevine red blotch-associated virus Is Widespread in the United States. Phytopathology 104, 1232-1240.

Kupferschmidt, K., 2013. A Lethal Dose of RNA. Science 341, 732 - 733.

Lindbo, J.A., Dougherty, W.G., 1992. Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. Virology 189, 725-733.

Lindbo, J.A., Silva-Rosales, L., Proebsting, W.M., Dougherty, W.G., 1993. Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. The Plant cell 5, 1749-1759.

Napoli, C., Lemieux, C., Jorgensen, R., 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. The Plant cell 2, 279-289.

Nunes, F.M.F., Aleixo, A. C., Barchuk, A. R., Bomtorin, A. D., Grozinger, C. M., and Simoes, Z. L. P, 2013. Non-target effects of green fluorescent protein (GFP)-derived double-stranded (dsRNA-GFP) used in honey bee RNA interference (RNAi) assays. Insects 4, 90 - 103.

Rosa, C., Kamita, S.G., Dequine, H., Wuriyanghan, H., Lindbo, J.A., Falk, B.W., 2010. RNAi effects on actin mRNAs in Homalodisca vitripennis cells. J RNAi Gene Silencing 6, 361-366.

Scorza, R., Callahan, A., Dardick, C., Ravelonandro, M., Polak, J., Malinowski, T., Zagrai, I., Cambra, M., Kamenova, I, 2013. Genetic engineering of Plum pox virus resistance: HoneySweet plum - from concept to product. Plant Cell Tiss Organ Cult.

Siomi, H., Siomi, M.C., 2009. On the road to reading the RNA-interference code. Nature 457, 396-404.
Tricoli, D., Carney, K. J., Russel, P. F., McMaster, J. R., Groff, D. W., Hadden, K. C., Himmel, P. T., Hubbard, J. P., Boeshore, M. L., and Quemada, H. D., 1995. Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to cucumber mosaic virus. Bio-Technology 13, 1458 - 1465.

PUBLICATIONS OR REPORTS RESULTING FROM THE PROJECT

1. Rosa C, Kamita, S. G., and Falk, B. W. 2012. RNA-interference is induced in the glassy-winged sharpshooter *Homalodisca vitripennis* by actin dsRNA. *Pest management science* Jul; 68 (7):995-1002.

2. Raja Sekhar Nandety, Almas Sharif, Tera L Pitman and Bryce W Falk. Glassy Winged Sharpshooter Insect Genome Footprints through Transcriptomic and Small RNA Sequence Profiling. Abstract presented at PAG conference, San Diego, CA, Jan 2013.

3. Raja Sekhar Nandety, Viacheslav Y. Fofanov, Heather Koshinsky, Drake C. Stenger and Bryce W. Falk. 2013. Small RNA populations for two unrelated viruses exhibit different biases in strand polarity and proximity to terminal sequences in the insect host *Homalodisca vitripennis*. Virology, 442:12-19.

4. Shizuo G. Kamita, Grant H. Oshita, Peng Wang, Raja Sekhar Nandety Christophe Morisseau , Bryce W. Falk and Bruce D. Hammock. 2013. Characterization of Hovi-mEH1, a microsomal epoxide hydrolase from the glassy-winged sharpshooter *Homalodisca vitripennis*. Archives of Insect Biochemistry and Physiology, 83 (4):173-179.

5. Raja Sekhar Nandety, Shizuo G Kamita, Bruce D Hammock and Bryce W Falk. 2013. Sequencing and de novo assembly of the transcriptome of the glassy-winged sharpshooter (*Homalodisca vitripennis*). PLoS One December 10, 2013, http://dx.doi.org/10.1371/journal.pone.0081681

6. Nandety, R. S., Kuo, Y.-W., Nouri, S., Falk, B. W. 2014. Emerging strategies for RNA interference (RNAi) applications in insects. Bioengineered, DOI:10.4161/21655979.2014.979701.

7. Nandety, R. S., Sharif, A., Kamita, S. G., Ramasamy, A., and Falk, B. W. 2015. Identification of novel and conserved microRNAs in *Homalodisca vitripennis*, the glassy-winged sharpshooter by expression profiling. PLoS One, I Oct 6;10(10):e0139771. doi: 10.1371/journal.pone.0139771.

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